CHEMICAL MODIFICATION AND BIOPHYSICAL STUDIES OF HEPATIC BOVINE FRUCTOSE-1,6-BISPHOSPHATASE

A THESIS

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BY

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sulfhydryl groups for activity are most likely located in a shallow pocket in the native enzyme.

DEDICATION

I wish to dedicate this thesis to my parents, Mr. Stanley G. and Shirley A. Brown, Sr., in appreciation for their many years of endless love and immeasurable support in all of my endeavors.

ACKNOWLEDGEMENTS

My most genuine and sincerest of gratitude I extend to my mentor and advisor, Dr. Henry Zeidan, whose unlimited patience and guidance has reinforced principles of persistency, dedication and resourcefulness. Also I would like to thank Dr. Peter Han, my co-advisor, whose generosity and willingness to help others has made my final passage within the University an ordered transition.

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ABBREVIATIONS

alpha	α
adenosine monophosphate	AMP
ammonium sulfate	$[(NH_4)_2SO_4]$
beta	β
circular dichroism	CD
5,5'-dithiobis (2-nitrobenzoic acid)	DTNB
ethylenediaminetetraacetic acid	EDTA
electron spin resonance	ESR
extinction coefficient	ε
fructose-1,6-bisphosphatase	FBPase
fructose-1,6-bisphosphate	FBP
fructose-6-phosphate	F-6-P
iodoacetic acid	IAA
N-ethylmaleimide	NEM
nicotinamide adenine dinucleotide phosphate	$NADP^+$
sodium dodecyl sulfate	SDS
spin label	SPL
sulfhydryl group	-SH

INTRODUCTION

The chemical modification of enzymes has been a major tool in the elucidation of enzymatic properties. Chemical modification studies have been used to pinpoint the nature of the active site residues and to differentiate between those amino acids that participate in the catalytic activity and those that are important in substrate binding. These investigations have also substantiated the existence of effector sites that control the overall reactivity of the enzyme molecule.

Once an enzyme molecule has been chemically altered, generally an analysis is performed to determine the efficacy with which the modified and native enzymes catalyze a particular reaction. The covalent transformation of amino acid side chains, particularly at the active site, is often manifested by dramatic changes in the specificity of the enzyme. Once this effect has been noted, it is then necessary to correlate cause with effect, i.e. to elucidate the identity of the amino acid that has been altered.

Fructose-1,6-bisphosphatase (FBPase) is a key enzyme in the pathway of gluconeogenesis. The enzyme, which is present in the liver, kidney, and skeletal muscle of mammals and other vertebrates, catalyzes the hydrolysis of fructose-1,6-bisphosphate (FBP) to yield fructose-6-phosphate (F-6-P) and inorganic phosphate (Pi).

The enzyme was first described by Gomori in 1943,[1] who separated it from other nonspecific phosphatases present in liver and kidney extracts and showed it to be inactive in the absence of magnesium (Mg^{2+}) ions. This absolute requirement for a divalent cation can also be satisfied by manganese (Mn^{2+}) ions.[2]

In 1961, progress on the physical and chemical properties of purified FBPase and its physiological function was sufficient to merit the organization of a conference devoted to this subject.[3] One of the most intriguing observations to emerge from the symposium was related to the pH optimum of liver FBPase. Reports from several laboratories suggested that under certain conditions, FBP was hydrolyzed at neutral pH, in contrast to the complete lack of activity below pH 8 which had been reported by Gomori.[1] Since that time, FBPase has been isolated from sources consisting of different mammalian tissues, plants, and microorganisms. Among these, the enzyme from rabbit liver has been more extensively investigated.

Unfortunately, studies on purified rabbit liver FBPase, prior to 1971, were limited to a modified form of the enzyme which showed an optimum activity at alkaline pH.[2] In 1971, Traniello, et al[4] reported the isolation of the native form of rabbit liver FBPase, which like the enzyme in crude extracts, showed an optimum activity at neutral pH. This "neutral" FBPase was found to have a higher molecular weight as compared with the previously isolated "alkaline" FBPase.[5,6] It is now known that the smaller molecular weight of the "alkaline" enzyme is the consequence of its hydrolysis by lysosomal proteases as the result of improper procedures of isolation.[7,8]

The neutral FBPases purified from rabbit liver and kidney were shown to be homogeneous proteins with a molecular weight of approximately 140,000.[4] This was significantly greater than the molecular weight of approximately 130,000 reported for the alkaline enzyme.[4] As determined by disk gel electrophoresis with sodium dodecyl sulfate, the neutral enzyme is a tetramer which dissociates to a single subunit type of 35,000 daltons.[9] This was in contrast to the alkaline enzyme preparations, which yielded two different subunits in disk gel electrophoresis, corresponding to molecular weights of approximately 36,000 and 30,000.

On the basis of amino acid composition and tryptic peptide maps, neutral rabbit liver, kidney, and intestinal FBPase are reported to be very similar.[10] In addition, similar data has been reported for the pig kidney enzyme.[11] The muscle enzyme, on the other hand, yields distinctly different peptide patterns.[12] Also published are the amino acid compositions of FBPase from rabbit liver (alkaline form),[13] mouse liver,[14] bovine liver,[15] and rabbit and chicken muscles.[16] Despite different amino acid compositions, there is a gross uniformity in the primary structure of the enzyme, since a given amino acid occurs either in relatively large, moderate, or low amounts in all FBPases.

As mentioned previously, purified liver FBPase shows an absolute requirement for a divalent cation which can be satisfied by Mn^{2+} ions or somewhat higher concentrations of Mg^{2+} ions. In the presence of Mn^{2+} ions the purified enzyme preparations have shown alkaline optima near pH 9, similar to those reported for the partially purified preparation by Gomori.[1] However, the shape of the pH activity curves was very much

dependent on the nature and concentration of the activating cation. With low concentration of Mg^{2+} ions maximum activity is also observed in the alkaline region, but at higher concentrations of this cation the pH optimum is shifted to the neutral pH range.[17]

The activation of FBPase by chelators in crude extracts had been reported earlier by a number of investigators. The activity of FBPase is increased when assayed in the presence of chelators such as histidine, imidazole,[18] citrate,[19] and EDTA[20] with the latter being the more effective. These effects are related to the removal of an inhibitory metal which has recently been identified as Zn^{2+} ions.[21,22]

Fructose-1,6-bisphosphatase is also activated by monovalent cations. More extensive studies have demonstrated that the enzyme from various vertebrate sources was activated by monovalent cations, with K^+ or NH_4^+ ions being the best activators.[23] It was shown that the presence of the monovalent cations altered the AMP-inhibition and Mg^{2+}-saturation curves for FBPase.

All mammalian FBPases are allosterically inhibited by AMP, and the inhibition depends on the temperature and on the concentration of Mg^{2+} ions.[24] Numerous kinetic studies have shown that the specific inhibition of AMP is noncompetitive in nature. An interesting feature of this inhibition by AMP, which is shared by other FBPases, is that it is less pronounced above pH 9.0. Recently, fructose-2,6-bisphosphate has been implicated as another potent inhibitor of FBPase,[25] and its action has been found to decrease with increasing temperature or pH. Its inhibition has been described as being competitive in nature and much more pronounced at low rather than at high substrate concentrations. Moreover, the products

of the reaction, fructose-6-phosphate and inorganic phosphate ion, are two other metabolities that regulate the activity of FBPase in a competitive manner.[26]

Fructose-1,6-bisphosphatase has been shown to exist in solution (pH 7.2, 25°C) as an equilibrium mixture of α-(15%) and β-(81%) furanose anomers, acyclic keto (2%) and gem-diol (1.3%) forms.[27] The enzyme, however, displays a stereospecificity for the α-anomer of fructose bisphosphate. The β-anomer is known to be utilized only after mutarotation.[28]

Sulfhydryl group modification studies on the activity of FBPase has been explored. Two groups have reported the altered catalytic and allosteric properties of bovine liver FBPase modified by several sulfhydryl reagents.[29,30] Although slightly varied experimental conditions gave an activated or inactivated enzyme with kinetic parameters changed in a complex manner, both groups observed four highly reactive thiol groups per molecule that were protected by high (>1 mM) concentrations of FBPase. Reaction of rabbit muscle and liver FBPase with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) showed four rapidly reacting sulfhydryl groups, a reduced rate of SH-group reactivity at high FBP concentrations, and a marked desensitization of modified enzyme to AMP inhibition.[31]

In summary, although chemical modification of FBPase does not abolish the turnover rate until more than four amino acid residues are involved, several clues have been uncovered as to the nature of the amino acids in the FBPase active site. Clearly, these studies indicate that one or more free cysteine residues may play an important role in FBPase catalysis.

EXPERIMENTAL

Materials

Livers from freshly sacrificed full-fed adult cows were obtained from Walker Meats in Carrollton, Georgia. d-Fructose-1,6-bisphosphate (FBP), nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, sodium iodoacetate, N-ethylmaleimide, phosphocellulose and other chemicals were all purchased from Sigma Chemical Company, St. Louis, Missouri, and Aldrich Chemical Company, Milwaukee, Wisconsin. The other common reagents used were of high quality grade.

Methods

Preparation of Resin

Phosphocellulose powder, the dry material obtained from commercial source, was first suspended in 0.5 N NaOH (1:20 vol) for 1 hr and the fine particles were removed by decanting several times. The suspension was then filtered on a coarse grade sintered glass funnel and washed free of acid with deionized water. This was followed by suspending the filtered cake again in 0.5 N NaOH, filtering and washing with deionized water free of alkali. The phosphocellulose was then treated with 20 vol of 0.25 N HCl for 30 min followed by distilled water washing until the effluent was near pH 7.0. Equilibration of the exchanger was followed by washing the phosphocellulose in an appropriate volume of 0.2 M sodium acetate buffer containing 0.1 mM EDTA, pH 6.3.

Assay Procedure

FBPase activity was measured spectrophotometrically in a coupled reaction by following the rate of $NADP^+$ reduction at 340 nm in the presence of excess of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase in a LKB spectrophotometer interfaced with an Apple IIe computer. The standard reaction mixture (1.0 ml) contained 50 mM Tris-HCl buffer (pH. 7.5), 0.1 mM $NADP^+$, 0.1 mM EDTA, 2 mM $MgCl_2$, 0.1 mM fructose-1,6-bisphosphate (FBP), 1 unit each of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, and an appropriate aliquot of purified bovine liver FBPase. One unit of enzyme activity was defined as the amount required to catalyze the hydrolysis of 1 μmole of FBP/min under these assay conditions. Specific activity is defined as units/mg of protein.

Alternatively, the activity of FBPase was assayed by measuring the release of phosphate ions according to the method of Tashima and Yoshimura.[31] The standard reaction mixture contained 0.1 mM FBP, 5 mM $MgCl_2$, 5 mM EDTA, and 50 mM Tris-HCl buffer, pH 7.2 in a final volume of 1.0 ml. The reaction was initiated by the addition of FBP and was carried out with gentle shaking at 25°C. It was terminated after 5 min by the addition of the color-developing reagent. After 15 min, the absorbance at 650 nm was determined and compared to values obtained with Pi standards. This method had the advantage that it did not require the addition of compounds other than the enzyme, substrate, activating cation and buffer.

Protein Determination

Protein concentration of the crude extract or partially purified enzyme was determined spectrophotometrically by the extinction coefficient at 280 nm (ε = 0.705).[32]

Polyacrylamide Disc Electrophoresis

The polyacrylamide disc electrophoresis was performed according to the method of Davis.[33] The different concentrations of polyacrylamide gels were prepared by mixing the appropriate amount of the gel buffer, acrylamide and bisacrylamide solution, ammonium persulfate, and water. The gel solution was then placed in the glass test tube, 10 cm long with an inner diameter of 6 mm, and a drop of water was added onto the top layer of the gel solution. The gel hardened after about 20 min, as indicated by the formation of an interface between the gel and water. About 20 µl containing 0.01 mg of the enzyme solution was mixed with 3 µl of 0.05% bromophenol blue in water and one drop of glycerol. The sample was then applied directly onto the gel. The top and bottom chambers contained diethylbarbituric acid-Tris buffer at pH 7.0. The electrophoresis experiment was conducted at a constant current of 6 mA/gel. The electrophoresis was stopped when the marker dye had travelled to the bottom of the gel. The gels were removed and stained with Coomassie blue for 2 hr. The destaining was done in 7.5% acetic acid solution containing 50% methanol and the destained gel was stored in 7.5% acetic acid solution. The mobility of the protein was calculated according to the following equation:

$$\text{MOBILITY} = \frac{\text{length before staining}}{\text{length after staining}} \times \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

Purification Procedure

Unless otherwise stated, all operations were carried out from 0-4°C. FBPase was purified from bovine liver by modification of the procedure of Han and Johnson.[32]

Step 1. Preparation of crude extract. Two-hundred (200) g of liver were cut into small pieces and homogenized in 600 ml of ice-cold 40 mM Tris-HCl buffer (ratio 1:3 w/v; pH 8.0) containing 1 mM EDTA, 10 mM cysteine, for 2 min at medium speed in a Waring blender. The homogenate was centrifuged for 40 min at 32,000 g, and the precipitate was discarded.

Step 2. Heat Treatment. The crude extract (pH 7.6) was transferred to two 500 ml beakers and heated with constant stirring in a water bath maintained at 85°C. When the temperature of the enzyme solution reached 68°C, the mixture was rapidly cooled in an ice bath and centrifuged to remove the precipitate.

Step 3. Removal of Unwanted Proteins by Phosphocellulose. The pH of the heat fraction was adjusted to 5.6, and the treated phosphocellulose corresponding to 30 g of dry weight was slowly added with constant stirring. During the addition of phosphocellulose, the pH of the solution was kept constant at 5.6. The deep red phosphocellulose was then removed by vacuum filtration. To the filtrate, another 30 g of treated phosphocellulose was added, maintaining the pH at 5.6. The phosphocellulose was again removed by filtration.

Step 4. Phosphocellulose Absorption and Chromatography. To the phosphocellulose treated fraction, FBP was added to achieve a final concentration of 2 mM. Phosphocellulose corresponding to 20 g of dry weight was added slowly maintaining the pH of the mixture at 6.3. The mixture was then

poured into a 600 ml coarse sintered glass funnel and the pH of the filtrate was adjusted to 7.4 with 3 M NaOH. To the pH adjusted filtrate, $MgCl_2$ was added to achieve a final concentration of 10 mM and the solution was allowed to stand for 20 min. More phosphocellulose was added to the mixture with the pH adjusted to 5.6 with 1.75 N acetic acid. After the suspension had settled, it was then filtered and washed with 0.2 M sodium acetate buffer, pH 6.3, containing 0.1 mM EDTA until all of the unabsorbed proteins were removed ($A_{280\ nm} = 0$).

The elution buffer containing 2 mM FBP and 0.1 mM 5'-AMP placed in the washing buffer mentioned above was used to elute the FBPase at a flow rate of 40 ml/hr. Fractions of 6 ml were collected and those exhibiting enzyme activity pooled. The enzyme was concentrated by $(NH_4)_2SO_4$ precipitation at 80% saturation, and was centrifuged for 20 min at 32,000 g. The enzyme was then stored in the cold.

Modification of FBPase

Modification of FBPase with iodoacetate, iodoacetamide, and N-ethylmaleimide was carried out under the conditions as described in the legends to figures. The number of reactive sulfhydryl groups per FBPase molecule before and after reaction with the N-ethylmaleimide and iodoacetate spin labels and reagents was determined by measuring the absorbance produced at 412 nm by reacting the enzyme with Ellman's reagent (DTNB) $\varepsilon = 13,600\ M^{-1}\ cm^{-1}$ for the reduced form of the native or spin labeled enzyme.

Amino Acid Analysis

Amino acid analysis were performed on 24 hr acid hydrolyzates (110°C), after the samples were passed through a 1.5 x 10 cm column of Sephadex G-25, on a Beckman Model 121 MB automatic amino acid analyzer.

Electron Spin Resonance

Electron spin resonance (ESR) measurements were recorded at room temperature on a Varian E-4 spectrometer.

RESULTS AND DISCUSSION

Purification of Bovine Liver FBPase

Fructose-1,6-bisphosphatase was purified as described previously by Han and Johnson.[32] The procedure was modified slightly as it is described in the Experimental section. This slight modification helped to speed up the purification and to increase the final yield by 30%. The modified steps introduced are the following: (a) 2-mercaptoethanol is replaced by cysteine in the homogenization buffer; (b) AMP and FBP are excluded in the preparation of the homogenization buffer. The result of a typical purification scheme is illustrated in Table 1.

Purity of the Enzyme

The purity of the enzyme was checked by polyacrylamide gel electrophoresis. The purified enzyme always showed a single band in the presence of sodium dodecyl sulfate (SDS) (Fig. 1). The molecular weight of the purified enzyme was determined from a standard curve of proteins of known molecular weight versus their electrophoretic mobility and was found to be 35,000 per monomer.

Modification of Enzyme with Iodoacetate, Iodoactemide, and N-ethylmaleimide

The enzyme was modified by reaction with either iodoacetate, iodoacetamide, or N-ethylmaleimide as described in the legends to Figures 2 and 3.

Table 1. Purification Scheme of Bovine Liver Fructose-1,6-Bisphosphatase

Step and Fraction	Total Units	Specific Activity (units/mg protein)	Yield
1. Crude Extract	1065	0.09	100
2. Heat Fraction	1021	0.32	96
3. Phosphocellulose-treated Fraction	1011	0.91	95
4. Phosphocellulose Eluate	729	25.1	69

The cysteine residues which are essential for activation or stimulation (one per subunit) reacted first with iodoacetate within 4 hr at pH 7.4 at 25°C. The catalytic activity of the enzyme was almost doubled after the first 4 hr (Fig. 2). Then the enzyme maintained its activity for more than 24 hr without detectable change. As the remaining essential cysteine residues for activity reacted, activity dropped progressively and after 48 hr about 15-20% of the activity remained.

The N-ethylmaleimide, however, reacted directly with the essential cysteine residues for activity (one per subunit or four per tetramer) with over 95% loss of activity of the enzyme occurring after 15 hr (Fig. 3).

From this study it was concluded that iodoacetate preferentially reacts first with the essential sulfhydryl groups required for activation and in doing so, changes the enzyme to its most reactive form. It then proceeds to react with the essential sulfhydryl groups necessary for activity or catalysis.

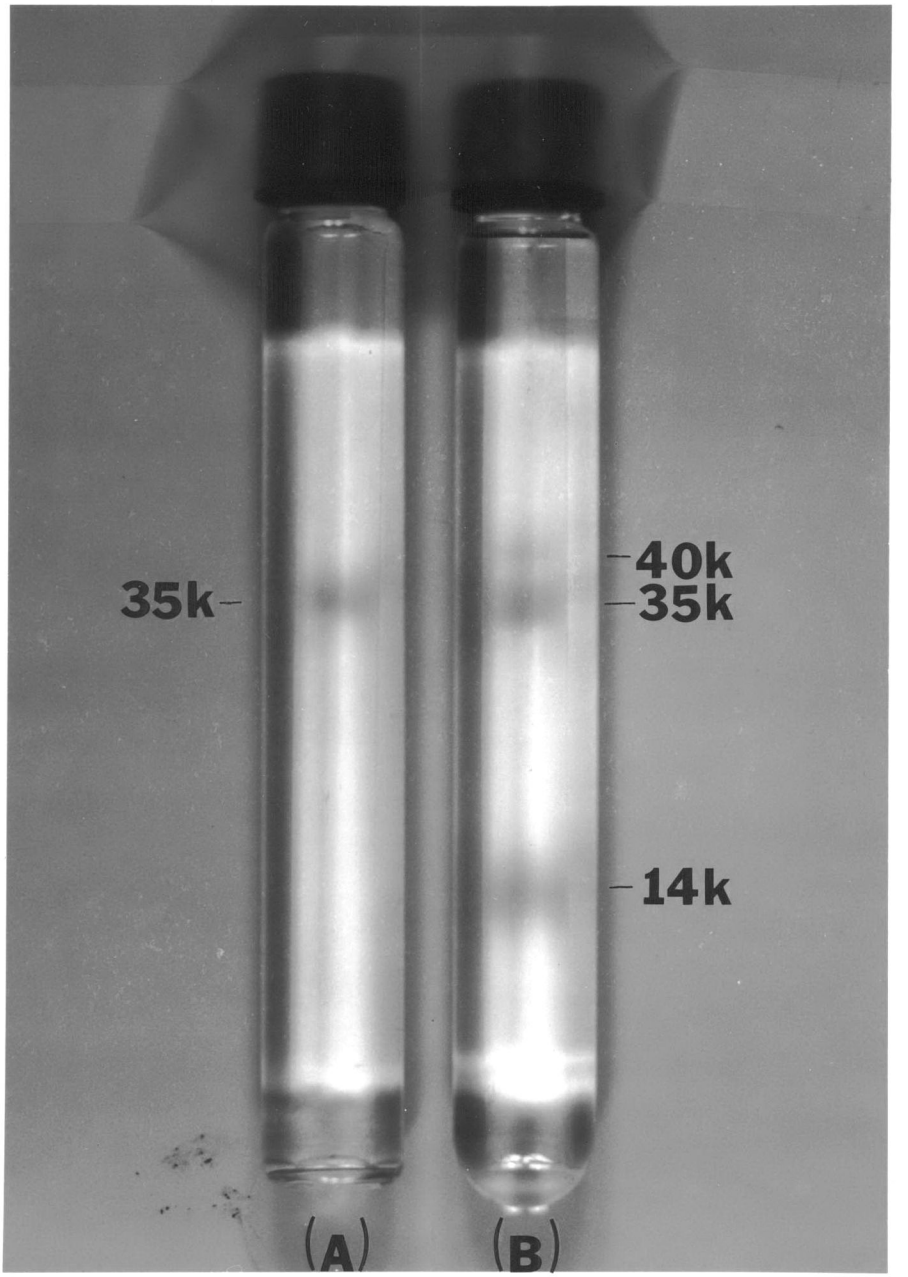

Figure 1. Polyacrylamide gel electrophoresis of purified bovine liver FBPase. (A) FBPase purified to homogeneity. (B) creatine mw, (40 K), FBPase mw (35 K), and lysozme mw (14 K) respectively.

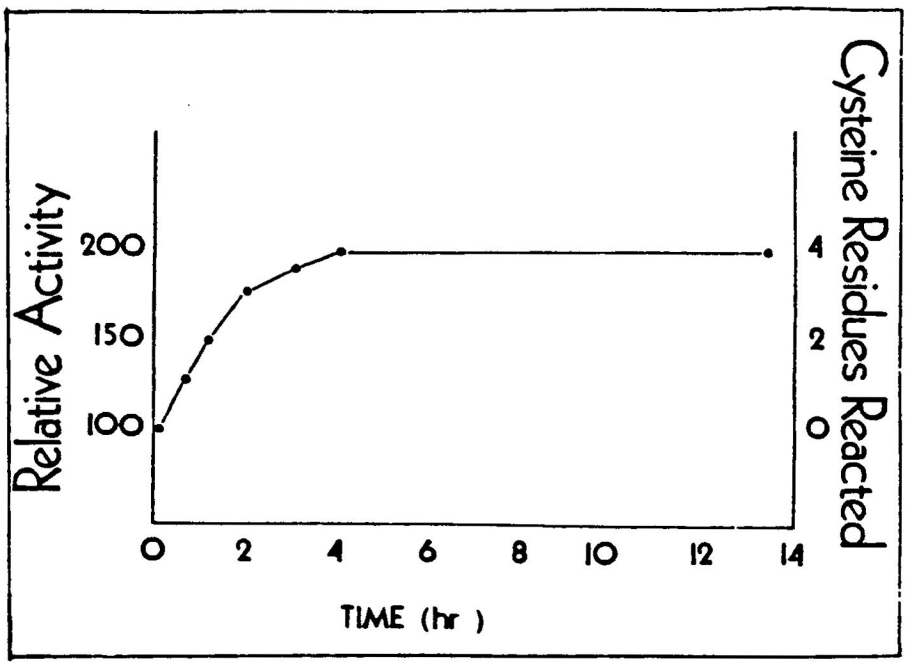

Figure 2. Stimulation of the catalytic activity of bovine liver FBPase by iodoacetate. The incubation mixture (1 ml) contained 100 mM sodium phosphate, 35 mM sodium iodoacetate, 0.1 mM EDTA, and 2 mg of enzyme in the final pH of 7.9. Incubation was initiated by the addition of enzyme and was carried out at 25°C. Aliquots were removed at the times specified and diluted with an appropriate amount of 50 mM Tris buffer (pH 7.5) containing 0.1 mM EDTA. The diluted enzyme was immediately assayed for enzyme activity as described in the Experimental section.

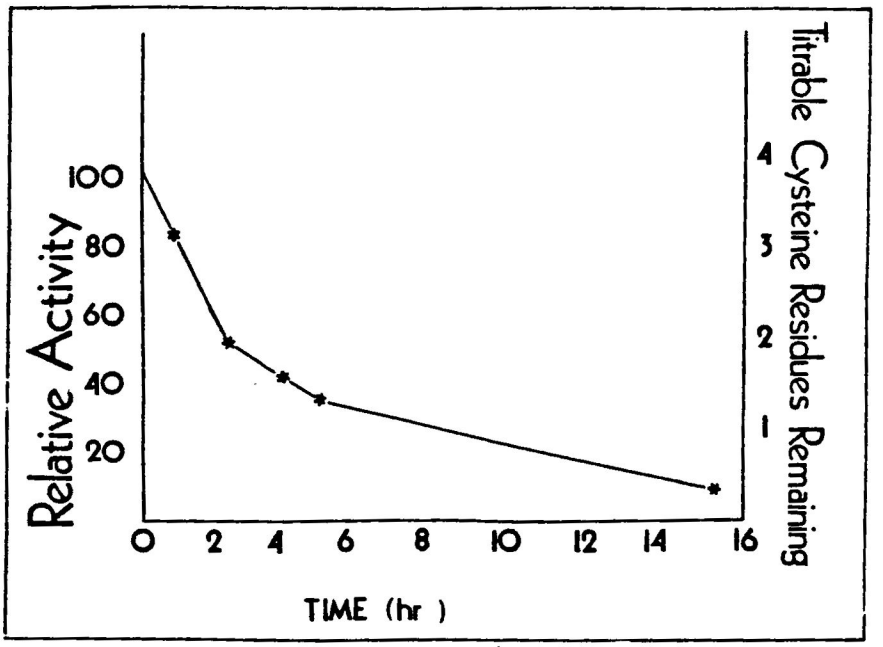

Figure 3. Inhibition of the catalytic activity of bovine liver FBPase by N-ethylmaleimide. The incubation mixture (1 ml) contained 100 mM sodium phosphate, 35 mM N-ethylmaleimide, 0.1 mM EDTA, and 2 mg of enzyme in the final pH of 7.9. Incubation was initiated by the addition of enzyme and was carried out at 25°C. Aliquots were removed at the times specified and diluted with an appropriate amount of 50 mM Tris buffer (pH 7.5) containing 0.1 mM EDTA. The diluted enzyme was immediately assayed for enzyme activity as described in the Experimental section.

On the other hand, treatment of the enzyme with N-ethylmaleimide immediately blocks those residues required for catalysis and abolishes enzymatic catalysis by 50% in a span of 2 hr. These results suggest the existence of two separate classes of sulfhydryl groups present within the enzyme. The first class is involved in the enhancement of enzymatic activity while the second is essential for catalysis.

Effect of Divalent and Monovalent Cations on Modified Enzyme

Figure 4 shows that treatment of bovine liver FBPase with 35 mM iodoacetate at pH 8.0 led to a gradual increase in enzyme activity. The increase in activity was more pronounced when the enzyme was assayed with Mn^{2+} than with Mg^{2+} ions as the essential divalent cation. After 3 hr of incubation, the activity was stimualted to the extent of approximately 150% or 350% depending on whether the enzyme was assayed with Mg^{2+} or with Mn^{2+} ions, respectively. Incubation beyond 3 hr was not found to further increase the activity significantly. This suggests that the essential sulfhydryl groups responsible for the activation of this enzyme had probably all been alkylated by iodoacetate within 3 hr of incubation.

Figure 5 shows that treatment with iodoacetate also resulted in the loss of the activation of the enzyme by K^+ or NH_4^+ ions when the enzyme was assayed with Mg^{2+}. The same results were subsequently found when Mg^{2+} was replaced by Mn^{2+} as the activating cation (data not shown). Although the modified enzyme used for studies shown in Fig. 5 had been treated with iodoacetate for 3 hr, the complete loss of the activation of the enzyme by univalent cations indeed occurred within 1.5 hr of incubation. This indicates that the complete loss of the activation of the

enzyme by K^+ or NH_4^+ ions occurred prior to the maximal stimulation of the enzyme activity during the course of treatment with iodoacetate. The basis for the loss of the activation of this enzyme by K^+ and NH_4^+ ions as the result of treatment with these two sulfhydryl reagents is not known. Two possibilities, however, are considered: (a) K^+ or NH_4^+ may activate this enzyme by electrostatic interaction with a specific -SH group which is susceptible to alkylation by iodoacetate; (b) K^+ or NH_4^+ is incapable of inducing more favorable enzyme conformation for catalysis after the enzyme is modified or activated by this sulfhydryl reagent.

It was found that the degree of activation of this enzyme by iodoacetate increased with increasing pH from 6.3 to 8.0 (see Table 2). Incubation of this enzyme with 35 mM iodoacetate at pH 5.4 in 100 mM sodium acetate buffer for 6 hr was not found to alter the catalytic activity significantly (data not shown). Since the activation of this enzyme by iodoacetate most likely results from the alkylation of some specific -SH groups, the increase in pH would produce more ionized species ($-S^-$) which should be more effective than the unionized species (-SH) in nucleophilic substitution reactions.

The altered properties of the enzyme induced by iodoacetate were not reversed after removal of nearly all reagent by extensive dialysis or by repeated precipitation with ammonium sulfate. This is consistent with the hypothesis that changes in enzyme properties by iodoacetate results from the formation of stable covalent bonds between carbon and sulfur atoms.

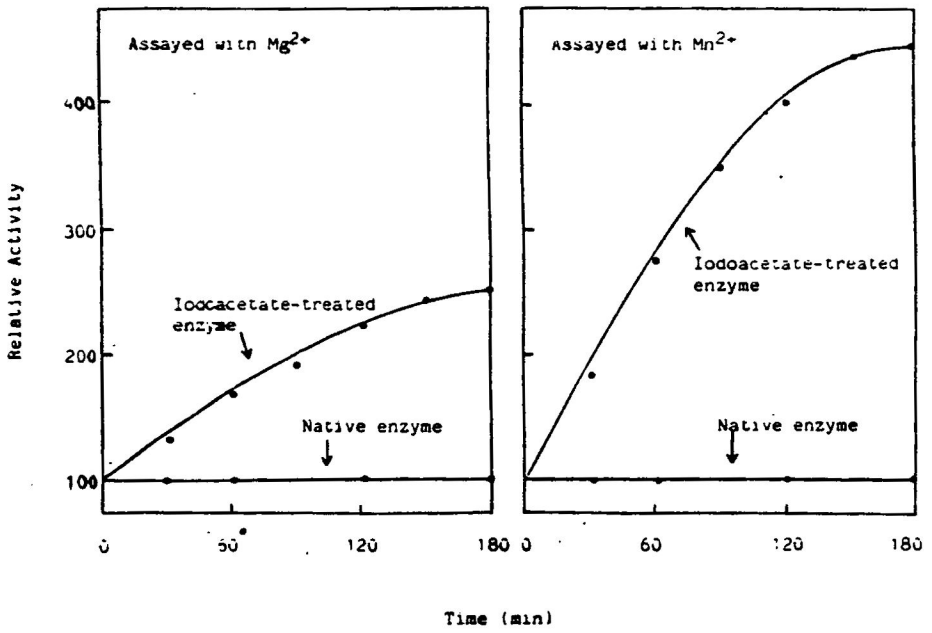

Figure 4. Stimulation of the catalytic activity of bovine liver FBPase by iodoacetate. The incubation mixture (1 ml) contained 100 mM sodium phosphate, 35 mM sodium iodoacetate, 0.1 mM EDTA, and 2 mg of enzyme in the final pH of 7.9. Incubation was initiated by the addition of enzyme and was carried out at 25°C. Aliquots were removed at the times specified and diluted with appropriate amount of 50 mM Tris buffer (pH 7.5) containing 0.1 mM EDTA. The diluted enzyme was immediately assayed for enzyme activity with either Mg^{2+} (Fig. 4a) or Mn^{2+} (Fig. 4b) as activating cation as described in the Experimental section. The relative activity of 100 is equivalent to the specific activity (μmoles/min/mg) of 9.2 or 11.3 tested with Mg^{2+} or Mn^{2+}, respectively.

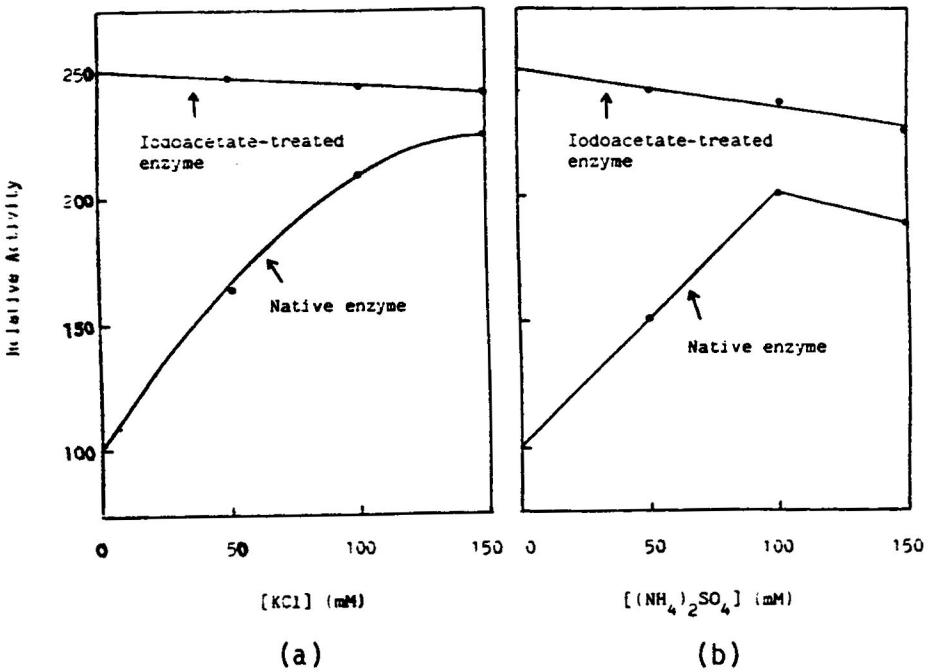

Figure 5. Effect of K^+ (Fig. 5a) and NH_4^+ (Fig. 5b) on the activity of native and iodoacetate-treated bovine liver FBPase. Treatment of FBPase with iodoacetate was performed as described in the legend to Figure 2. After incubation for 3 hr, samples of both the treated and untreated FBPase were removed and diluted with an appropriate amount of 50 mM Tris buffer (pH 7.5) containing 0.1 mM EDTA. They were immediately assayed for enzyme activity with Mg^{2+} as activating cation and at various concentrations of KCl or $(NH_4^+)_2SO_4$ as indicated.

Table 2. Effect of pH on the Stimulation of the Catalytic Activity of Bovine Liver Fructose-1,6-Bisphosphatase

Conditions of Treatment		Specific activity (μ moles/min/mg) Conditions of assay[a]	
pH	Time (min)	With Mg^{2+}	With Mn^{2+}
6.3	0	9.2	11.1
	120	10.1	11.9
7.2	0	9.3	11.4
	120	14.3	20.6
8.0	0	9.2	11.3
	120	19.3	32.1

[a]The incubation mixture (1 ml) contained 100 mM sodium phosphate, 35 mM sodium iodoacetate, 0.1 mM EDTA, and 2 mg of enzyme. Incubation was initiated by the addition of enzyme and was carried out at 25°C. Samples were collected immediately after addition of enzyme and after 120 min, were diluted with an approximate amount of 50 mM tris buffer (pH 7.5) containing 0.1 mM EDTA. This was assayed for enzyme activity with Mg^{2+} or Mn^{2+} as activating cation as described in the Experimental section.

ESR Studies of Spin-Labeled Iodoacetamide and Spin Labeled N-ethylmaleimide

The enzyme was reacted with spin labeled iodoacetamide for 4 hr and the spectrum obtained is shown in Fig. 6. The reporter group is strongly immobilized with a splitting constant of 64 gauss which is indicative of cysteine residues buried in a deep pocket. This data, along with chemical modification studies of the enzyme by iodoacetate, clearly suggest that the first class of sulfhydryl groups is located in a deep pocket.

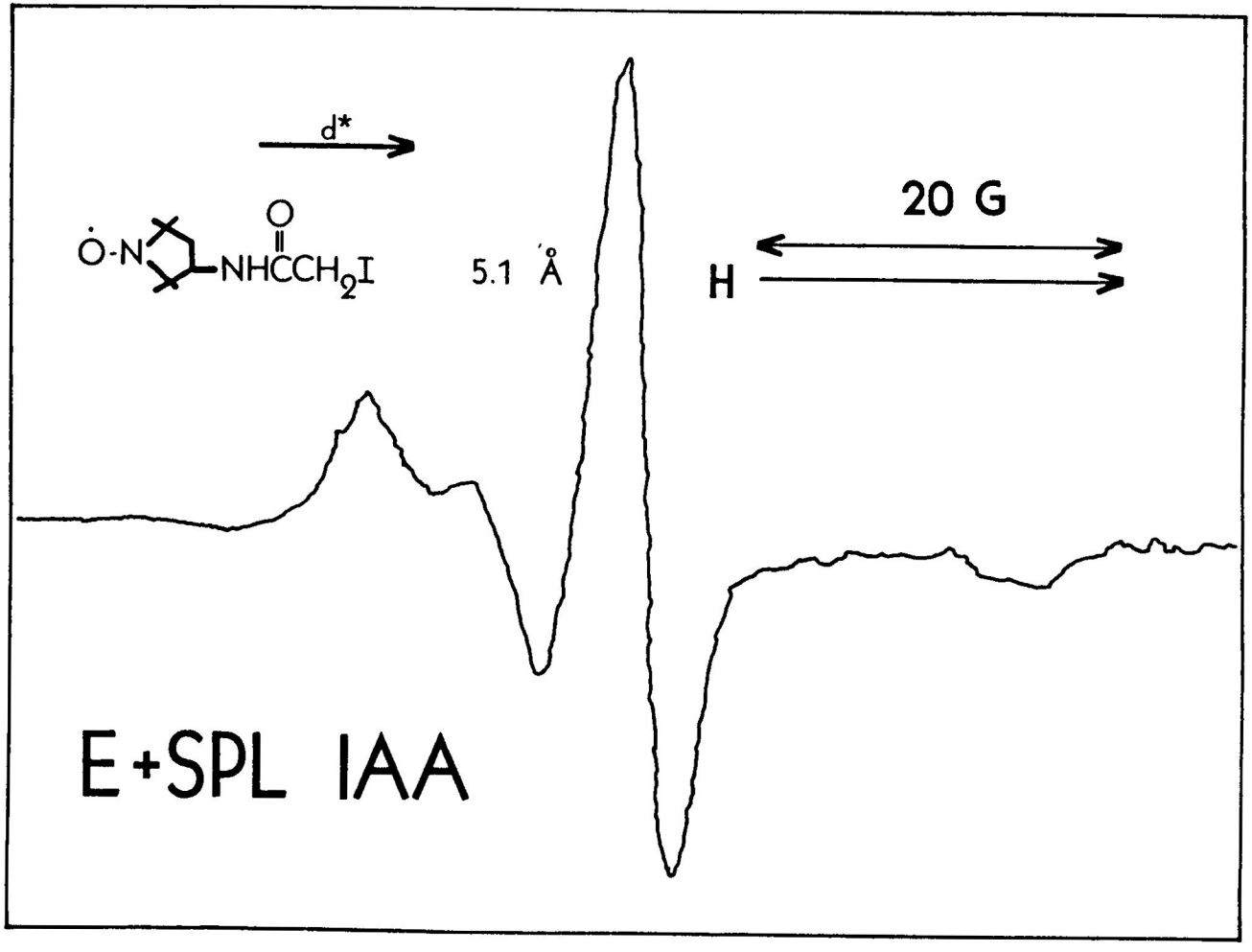

Figure 6. ESR spectrum of spin labeled fructose-1,6-bisphosphatase with spin label iodoacetamide.

*d is the length of the side chain.

In another experiment the enzyme was reacted with spin labeled N-ethylmaleimide for a period of 6 hr. The ESR spectrum as shown in Fig. 7 exhibits two different types of immobilization, one strongly and one partially immobilized label. The ESR spectral data (Fig. 7) along with the chemical modification of the enzyme with N-ethylmaleimide suggests that the second class of sulfhydryl groups is located in a shallow pocket.

Amino Acid Analysis

Amino acid analysis of the enzyme and its derivatives, as shown in Table 3, suggest that none of the histidine or lysine residues are carboxymethylated under these conditions.

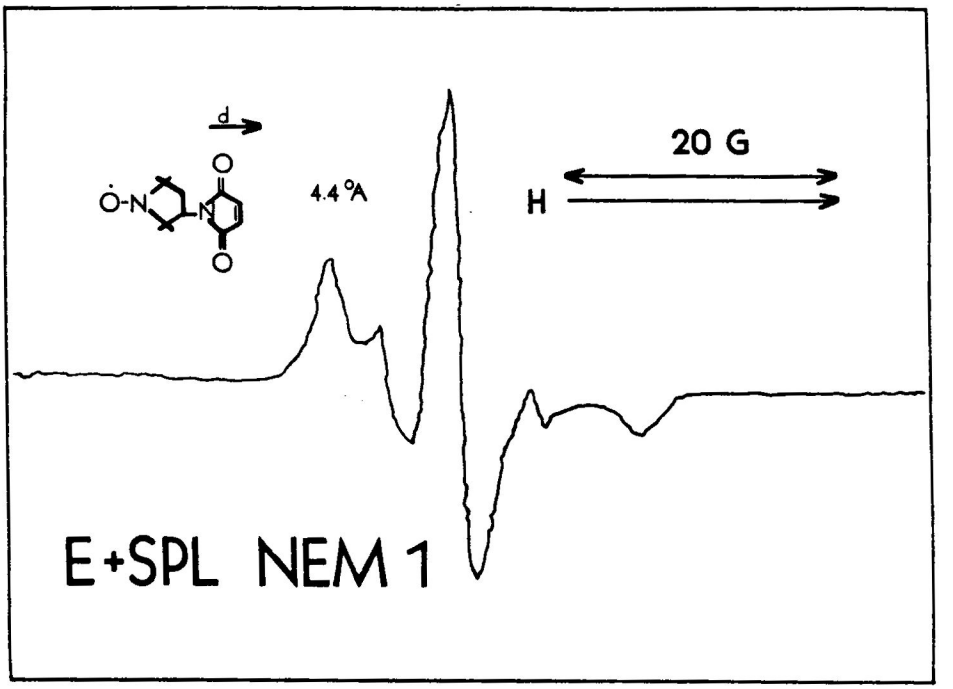

Figure 7. ESR spectrum of spin labeled fructose-1,6-bisphosphatase with spin label N-ethylmaleimide.

*d is the length of the side chain.

Table 3. Amino Acid Analysis of Bovine Liver Fructose-1,6-Bisphosphatase

Amino Acid	Sample Fraction*				
	Control	E+IAA	E+NEM	E+IAA +SPL IAA	E+IAA +SPL NEM
Alanine	28	29	28	30	30
Aspartic Acid	28	28	24	28	31
Glutamic Acid	21	21	18	21	24
Phenylalanine	11	11	12	11	10
Glycine	32	34	33	36	41
Histidine	04	03	05	06	06
Isoleucine	25	24	25	24	22
Lysine	23	23	26	21	26
Leucine	28	27	27	28	26
Proline	15	16	16	16	16
Arginine	15	16	16	16	16
Serine	22	22	22	28	34
Threonine	20	20	20	20	21
Valine	28	29	29	29	28
Tyrosine	17	16	16	16	14

*Shown are the estimated number of residues per peptide. Values indicate the actual recovery of peptides in pm of amino acid residue.

CONCLUSION

In summary, we can conclude the following:

1. Modification of the enzyme with different sulfhydryl agents demonstrates the existence of two different classes of cysteine groups. The first is essential for activation and can be explored by reacting the enzyme with iodoacetate or iodoacetamide; whereas the second class, which is directly essential for activity and catalysis, can be explored by reacting the enzyme with N-ethylmaleimide.
2. Modification of bovine liver FBPase with iodoacetate leads to a marked increase in enzyme activity. This activation is much more pronounced when the enzyme is assayed with Mn^{2+} than with Mg^{2+} ions as the activating divalent cation.
3. Treatment with iodoacetate also leads to the loss of the activation of the enzyme by univalent cations (K^+ and NH_4^+).
4. The degree of modification of the enzyme by iodoacetate increases significantly with increasing pH, and the loss of activation altered properties of the enzyme by univalent cations (K^+ and NH_4^+) increases significantly with increasing pH.
5. The essential sulfhydryl groups for activation are located in a deep cleft whereas the essential sulfhydryl groups for activity are most likely located in a shallow pocket in the native enzyme.

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